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(54) Title: NOVEL POLYPEPTIDE SCAFFOLDS AND USE THEREOF

(57) Abstract: Novel peptide having a sequence according to SEQ. ID. No.1, SEQ. ID. No. 2 and/or SEQ. ID. No. 3 are disclosed and also polypeptide scaffold consisting of a four helix bundle formed of two dimerized helix-loop-helix motifs, said helix-loop-helix motifs having sequences according to SEQ. ID. No. 1, SEQ. ID. No. 2 and/or SEQ. ID. No. 3 which may comprise a fluorescent probe at the side chain of Lys15 and a ligand with affinity for a target molecule at the side chain of Lys8 or Lys34. Also disclosed are polypeptide scaffolds for use in biosensing applications with or without the polypeptide scaffold anchored to a solid surface.

NOVEL POLYPEPTIDE SCAFFOLDS AND USE THEREOF

Field of the invention

The present invention relates to novel polypeptide scaffolds and use thereof.

Background of the invention

The *de novo* design of folded polypeptides aims at improving our understanding of protein structure, and also provides a platform for the engineering of new proteins with tailored functions [DeGrado, W. F.; Summa, C. M.; Pavone, V.; Nastri, F.; Lombardi, A. *Annu. Rev. Biochem.* **1999**, *68*, 779-819; Micklatcher, C.; Chmielewski, J. *Curr. Opin. Chem. Biol.* **1999**, *3*, 724-729; Baltzer, L.; Nilsson, H.; Nilsson, J. *Chem. Rev,*101,3153-3164. Designed, folded polypeptides that undergo pH-controlled, site selective self-functionalization with ligands [Broo, K.; Brive, L.; Lundh, A. C.; Ahlberg, P.; Baltzer, L. *J. Am. Chem. Soc.* **1996**, *118*, 8172-8173; Baltzer, L.; Nilsson, J. *Curr. Opin. Biotechnol.* **2001**, *12*, 355-360] constitute an excellent toolbox for the construction of various complex molecular systems, e. g. model glycoproteins [Andersson, L.; Stenhagen, G.; Baltzer, L. *J. Org. Chem.* **1998**, *63*, 1366-1367; Andersson, L. K.; Dolphin, G. T.; Kihlberg, J.; Baltzer, L. *J. Chem. Soc.-Perkin Trans. 2* **2000**, 459-464] or complex receptors.

Summary of the invention

The object of the present invention is to provide folded, ligand modified helix-loop-helix polypeptide scaffolds that connect the key biosensing events of recognition and reporting. The well characterized interaction between the enzyme human carbonic anhydrase II, HCAII, and its inhibitor 4-carboxybenzenesulfonamide [Vidgren, J.; Svensson, A.; Liljas, A. *Int. J. Biol. Macromol.* **1993**, *15*, 97-100] (**Ia**) was selected for a proof of principle demonstration. However, the variety of molecules that can be incorporated, and the ease by which their relative positions can be varied, allow for functional units for a wide range of receptor-ligand systems to be systematically developed.

More precisely, the present invention relates to novel peptides having a sequence according to SEQ. ID. No. 1, SEQ. ID. No. 2 and/or SEQ. ID. No. 3.

These peptides constitute helix-loop-helix motifs. The helix-loop-helix motif having SEQ. ID. No. 2 is shown as KE2 in figure 6, and the helix-loop-helix motif having SEQ. ID. No. 3 is shown as KE3 in figure 6.

The invention also relates to polypeptide scaffold consisting of a four helix bundle formed of two helix-loop-helix motifs, i.e. two of the above mentioned peptides, which have dimerized.

Furthermore, the invention relates to the use the above mentioned polypeptide scaffolds in bioanalytical/biosensor applications.

Finally, the invention relates to the use of such polypeptide scaffolds in biosensors for determination of protein concentrations and/or protein affinities.

The characterizing features of the invention will be evident from the following description and the appended claims.

Detailed description of the invention

Preferred polypeptide scaffold according to the invention are SEQ. ID. No1, KE2 (SEQ. ID. No. 2) and KE3 (SEQ. ID. No. 3) bound to a polypeptide sequence that makes them form a helix-loop-helix motif. Thus KE2 may dimerize with a second copy of KE2 to form a four-helix bundle, and KE3 may dimerize with a second copy of KE3 to form a four-helix bundle. KE2 may also dimerize with KE3, to form a heterodimer or with SEQ. ID. No1 or another sequence based on that of KE2 or KE3, and that have the same hydrophobic residues in the same positions in the sequence and form the helix-loop-helix motif.

The polypeptide scaffold according to the invention preferably comprises a ligand with affinity for a target molecule or ion. By "target molecule or ion" is intended a molecule or ion to which said ligand binds for the purpose of detection and quantification. For example, if the polypeptide scaffold has been functionalized with a ligand that specifically binds a metal ion the purpose is to detect and quantify that "target" metal ion. If the polypeptide scaffold has been functionalized with a ligand with high specificity for a protein the purpose is to identify and quantify that "target" protein. Targets include , but are not limited to metal ions and proteins. Interesting target molecules are, e.g., biomolecules. Preferably said ligand is localized at the side chain of a lysine residue. In KE2 this lysine residue preferably is Lys34 because it is preferentially acylated due to its low pKa value and in KE3 this lysine residue is Lys8 because it is close to the His residue in position 11 which ensures the site selectivity. The choice

of the ligand depends on the intended use of the polypeptide scaffold. For detection of enzymes the ligand is chosen from their known inhibitors, for detection of proteins other than enzymes, that have high affinity ligands, the ligand to be attached to the scaffold is chosen from the known ligands of the protein. For carbohydrate binding proteins the ligand is a carbohydrate. For DNA and RNA the ligand is DNA, RNA or PNA. For target proteins for which there are no known ligands, the ligands to be attached to the scaffold are several compounds from a combinatorial library. One example of such a ligand is benzenesulfonamide which is an inhibitor of carbonic anhydrase II. Any thrombin inhibitor can be used for the detection of thrombin and protease inhibitors can be used for the detection of proteases. For detection of ions the ligand is an ion binding functionality or chelating group.

The polypeptide scaffold according to the invention preferably also comprises a reporter group, or reporting group, for enabling detection of the binding to the ligand. Identification and quantification of a target ion is for example of interest in the study of environmental pollution or in process control. Identification and quantification of biomolecules such as metabolites or proteins are of interest in the diagnosis and treatment of disease. The reporter group can e.g. be a fluorescent probe. This fluorescent probe may be e.g. dansyl, fluorescein, rhodamine or Oregon Green derivatives. Preferably it is attached to the side chain of Lys15. The positions of the ligand and the fluorescent probe may be reversed in each polypeptide.

The ligand and the reporter group may either both be attached to the same polypeptide chain or be attached to different polypeptide chains of the dimer.

Two or more polypeptide scaffolds may be arranged e.g. in the form of an array on a biosensor chip or in the wells of a microtiter plate. When a microtiter plate is used, the polypeptide scaffolds may be present in a solution or in a polymeric hydrogel in the wells of the plate. It may also be possible to use a solution in indentations, pits or cavities on the chip or in the wells of the microtiter plate. The chip can be made of metal, insulator, semiconductor or polymer. The chip can also consist of a thin coating of the above mentioned materials, e.g. in the form of an array, on top of a supporting substrate.

It is often preferable to immobilize the polypeptide scaffolds on the surface of e.g. a chip, a microtiter plate, a vesicle, a micelle, or a membrane. Examples of such surfaces are artificial surfaces such as modified or unmodified

surfaces of a metal, an insulator, a semiconductor or a polymer. Other examples of surfaces to which the polypeptide scaffoldes may be immobilized are surfaces of vesicles, micelles and membranes. It may be preferable to include an anchoring group in the polypeptide scaffold according to the invention. One way of doing this is to introduce an amino acid with a high affinity for the surface onto which the polypeptide scaffolds are to be immobilized, such as a chip or microtiter plate or a vesicle or membrane. One example of doing this is to introduce at position 22 of the loop region a Cys, Lys or Glu residue or a non-natural amino acid. The site of introduction is not limited to position 22, but may be any position in the loop region in positions 20-24 of the sequence. The non-natural amino acid may e.g. have an aminooxy function. Another way is to attach a bifunctional linker molecule to an amino acid residue in the polypeptide. Preferably, the anchoring group has been introduced site-specifically into the polypeptide scaffold by attachment of a bifunctional molecule to an amino acid residue specifically introduced to react chemoselectively or site-selectively with the bifunctional linker molecule according to the principles described above.. The introduced amino acid or linker molecule should be able to form a strong chemical bond to the surface onto which the polypeptide scaffolds are to be immobilized, such as a chip or microtiter plate or a vesicle or membrane, and they could be of the type –SH, COOH, NH_2, biotin, his-tag, fatty acid, cholesterol etc. The bifunctional molecule may have the general structure X-R_n-Y, wherein X is a functional group of the type COOH, NH_2, SH, SSAr, CHO, CH_2Br, CH_2Cl, or CH_2I, R_n is an alkyl or ethylene glycol chain comprising n carbons, and Y is a group of the type COOH, NH_2, SH, biotin, biotin analogue, His-tag, fatty acid or cholesterol.

The binding of the polypeptide scaffold can occur either directly to the substrate surface onto which the polypeptide scaffolds are to be immobilized, such as a chip or microtiter plate or a vesicle or membrane, or indirectly via a surface modification formed on the surface onto which the polypeptide scaffolds are to be immobilized, such as a chip or microtiter plate or a vesicle or membrane. The surface modification can be a self-assembled monolayer, a protein layer (e.g. streptavidin), a polymer network, a hydrogel, a His-tag etc.

A cysteine residue introduced into the polypeptide scaffold can react via the SH group directly with the surface, e.g. gold. A cysteine residue introduced in the same way also can react via the SH group with a disulphide attached to the surface, Figure 9. This latter procedure is only meant as an illus-

tration and is explained in figure 8, but many other methods exist which will achieve the same purpose.

The polypeptide scaffold according to the invention is highly suitable for determination of protein concentrations and protein affinities in biosensing applications. It can also be used for the determination of DNA, RNA and PNA concentrations and affinities, as well as carbohydrate concentrations and affinities.

In the determination of protein concentrations or affinities polypeptide scaffolds may be used in arrays where the ligands of the polypeptide scaffolds have the same or different affinities for the protein or other substance that is to be studied.

When polypeptide scaffolds having different affinities are used in arrays for biosensing applications they are suitable for measurements of analyte concentrations, and when polypeptide scaffolds having the same affinity are used in arrays for biosensing applications they are suitable for measurements of the affinity of the analyte for the ligand used through the use of different dilutions of the sample for the different spots of the array. It is for example of interest to be able to determine the concentrations of proteins that are related to a disease, and to be able to determine the affinities of those proteins. Altered concentrations and affinities may suggest a pathological condition or the absence of a pathological condition. It is also of interest to be able to monitor the concentrations and affinities of proteins to prevent disease, and to predict the probability of disease. It is also of interest to determine the concentration of m-RNA, or carbohydrates or other biomolecules.

The invention will now be further explained in the following example. The example is only intended to illustrate the invention and should in no way be considered to limit the scope of the invention.

Brief description of the drawings

In the examples, reference is made to the accompanying drawings on which:

Figure 1 shows a modeled structure of KE2 and KE3, showing sites of introduction of dansyl, position 15, and benzenesulfonamide, positions 34 and 8, respectively. Only the amino acid side chains in positions involved in functionalization are shown and only those in the sequence of KE2. Amino acid

sequences of KE2 and KE3, where lysine residues in bold represent sites of modification, are also shown.

Figure 2 shows a fluorescence spectrum of 1 μM KE2 (upper) and KE3 (lower) modified with the dansyl fluorescent probe. Bold and thin curves correspond to peptides with and without benzenesulfonamide, respectively (KE2-PL, KE3-PL and KE2-P, KE3-P). Dotted lines represent spectra in the absence of HCAII, solid lines represent spectra where 50 μM HCAII has been added. The fluorescence of the control peptides KE2-P and KE3-P is not affected by the presence of HCAII, but the fluorescence of KE2-PL is increased by 80%. KE3-PL displays a similar, but less pronounced, behavior. Note the significant difference between the fluorescence of KE3-PL and KE3-P.

Figure 3 shows a sigmoid binding curve obtained upon titration of 1 μM KE2-PL with HCAII by plotting the maximum fluorescence intensity versus the logarithm of free HCAII concentration. The affinity of the interaction was estimated to be 0.02 μM from curve fitting using a two-state binding model.

Figure 4 shows mean residue ellipticity of 1 μM KE2-PL (solid curve) and 1 μM KE2-PL in the presence of 2 μM HCAII (dotted curve). The dotted curve is the difference spectrum obtained by subtracting the spectrum of 2 μM HCAII from that of 1 μM KE2-PL + 2 μM HCAII. The spectrum of KE2-PL shows two minima at 208 and 222 nm that are typical of α-helical proteins. The helicity of the peptide was retained upon binding to HCAII.

Figure 5 shows the affinity of the interaction between KE2-PL and HCAII was estimated to be 0.08 μM from a surface plasmon resonance-based analysis. The solid line represents the theoretical curve for equilibrium response as a function of peptide concentration with K_d = 0.08 μM (bimolecular interaction model). Experimental data from duplicate measurements are shown.

Figure 6 shows the sequences of KE2 and KE3, and also of LA-42b.

Figure 7 shows the structures of compounds **Ia**, **Ib**, **Ic** and **Id**.

Figure 8 illustrates the procedure of immobilization via PDEA where a carboxylate residue linked to the gold surface of a Biacore chip is transformed into an N-hydroxysuccinimide (NHS) ester using EDC as coupling agent, and reacted with PDEA to form the corresponding amide. The Cys residue of the peptide KE3 was then reacted with the PDEA moiety to form the covalent disulfide bond.

Figure 9 shows a Biacore sensorgram describing the immobilization of KE3 in dextran matrix and on 100% carboxylate-presenting self assembled monolayer (SAM)using the procedure of Figure 8.

Example

The design of the polypeptides KE2 and KE3 (see Figure 1) was based on the sequence of LA-42b, a 42 residue polypeptide that folds into a helix-loop-helix motif and dimerizes to form a four helix bundle [Andersson, L.; Stenhagen, G.; Baltzer, L. *J. Org. Chem.* **1998**, *63*, 1366-1367] (shown in Figure 6 and as SEQ. ID. No. 4). Out of 42 residues of LA-42b more than 32 were conserved in the design of KE2 and KE3. The solution structure of LA-42b has been extensively studied by NMR and CD spectroscopy and because of the sequence similarities with KE2 and KE3 they, too, were assumed to fold into helix-loop-helix dimer motifs in aqueous solution. They were synthesized using solid phase peptide synthesis and identified by mass spectrometry. The MALDI-TOF spectra of KE2 and KE3 showed single peaks at 4446.3 and 4563.2, respectively (calc. 4446.0 and 4563.2). The peptides were designed to allow the site-specific incorporation of a fluorescent probe at the side chain of Lys15, as well as of a ligand with high affinity for a target protein at the side chains of Lys34 (KE2) or Lys8 (KE3). The side chain of Lys15 was orthogonally protected to allow the coupling of a fluorescent probe on the solid-phase. Before cleaving the peptide from the resin the Lys15 Alloc protection group was removed by three equivalents of Pd $(PPh_3)_4$ in a mixture of 9.25 mL $CHCl_3$, 0.5 mL AcOH and 0.5 mL N-methylmorpholine. Reaction of the selectively deprotected peptides with two equivalents of dansyl chloride in the presence of eight equivalents of diisopropylethylamine in DMF provided KE2-P and KE3-P. The MALDI-TOF spectra of KE2-P and KE3-P showed single peaks at 4680.4 and 4868.2, respectively (calc. 4679.4 and 4867.6). The notation –P indicates that a fluorescent probe has been covalently attached and the notation –PL indicates the attachment of both fluorescent probe and high-affinity ligand.

The incorporation of the benzenesulfonamide ligand was accomplished by reacting the polypeptides with the active ester **Id** in aqueous solution at pH 8. One of the inventors et al have previously shown that Lys34 is the most reactive of all lysine residues in LA-42b [Andersson, L. K.; Caspersson, M.; Baltzer, L. Chem. Eur J. 2002, In press] in terms of its reactivity towards ac-

tive esters, because it is situated in a position that forms a part of the hydrophobic core, and has a selectively depressed pK_a value. Some competition from Lys19 was observed [Andersson, L. K.; Dolphin, G. T.; Baltzer, L. ChemBioChem, 2002, in press] and therefore Lys19 was replaced by Arg19 in the sequence of KE2, and so was Lys10. No competition from Lys33 was expected based on the previous investigation and it was therefore not removed. In KE3 Lys8 is less reactive than Lys34 and Lys19, and only equally reactive as Lys10 and Lys33, and consequently in KE3 all competing lysines were replaced by Arg residues.

The affinity of HCAII for unprotonated **Ia** in aqueous phosphate buffer at room temperature and pH 6.5 has been reported previously, and the equilibrium dissociation constant K_d was 27 μM. [Taylor, P. W.; King, R. W.; Burgen, A. S. V. *Biochemistry* **1970**, *9*, 2638-2645] Preliminary surface plasmon resonance-based affinity measurements suggested that K_d for KE2-PL modified with **Ib** was in the μM range and the inventors reasoned that in order to avoid competition from non-specific binding of HCAII a higher affinity ligand than that corresponding to **Ia** was needed. With benzenesulfonamide derivatives bearing alkyl chains of different lengths in the *para* position, increased affinities towards HCAII as compared to that of **Ia** have been reported [King, R. W.; Burgen, A. S. V. *Proc. R. Soc. Lond. B* **1976**, *193*, 107-125]. Thus, to increase the affinity of the benzenesulfonamide inhibitor, and to minimize sterical constraints upon HCAII binding that could be introduced by coupling the benzenesulfonamide ligand to the peptide, an aliphatic spacer was introduced. The *N*-hydroxysuccinimidyl ester of 4-carboxybenzenesulfonamide (**Ib**) was reacted with 6-aminohexanoic acid to form **Ic**, which was further activated with *N*-hydroxysuccinimide to form **Id**. 1.4 equivalents of **Id** were allowed to react with 1 equivalent of KE2-P or KE3-P in 50 mM Tris-HCl buffer at pH 8.0 and room temperature. The modified peptides KE2-PL and KE3-PL were purified by reversed-phase HPLC on a Hichrom C-8-column using 0.1% TFA in 40% aqueous 2-propanol as the eluent and lyophilized. The yield was 55% and 77% for KE2-PL and KE3-PL, respectively. The MALDI-TOF spectra of KE2-PL and KE3-PL showed single peaks at 4977.2 and 5165.0, respectively (calc. 4975.7 and 5163.9).

The biosensing capabilities of KE2-PL and KE3-PL were investigated by recording their fluorescence emission spectra between 450 and 650 nm upon excitation at 335 nm. Spectra were recorded of 1 μM peptide solutions in

10 mM HBS buffer at pH 7.4 and 298 K, in the absence and presence of 50 μM HCAII, with 1 μM solutions of KE2-P and KE3-P as negative controls, Figure 2. Upon addition of HCAII, the fluorescence intensities of KE2-PL and KE3-PL increased by 80% and 60%, respectively, whereas the fluorescence intensities of the control peptides were not affected by HCAII. The observed intensity increases were thus caused by the binding of HCAII to the benzenesulfonamide moiety of the peptide scaffolds, with negligible effects induced by non-specific protein-peptide interactions. The inventors interpret the intensity increases to arise from a change in molecular environment of the dansyl group upon binding of HCAII by the polypeptides; it appears that the probe is partially quenched in the unbound peptide, but less so when bound to HCAII. The polypeptides KE2-PL and KE3-PL are therefore capable of reporting on the presence of the target protein HCAII. Recognition is ensured by the specificity of the benzenesulfonamide ligand.

Upon titration of 1 μM KE2-PL with 5 nM – 50 μM HCAII, a sigmoid curve was obtained, Figure 3. For the bimolecular association between KE2-PL and HCAII, the equilibrium dissociation constant K_d equals the concentration of free HCAII at the inflexion point. K_d was estimated to be 0.02 μM from the best fit to the experimental results of an equation describing the dissociation of a bimolecular complex. This result constitutes the proof of principle for functional helix-loop-helix-based biosensor units, since binding results in fluorescence intensity changes. The use of an array of peptides modified with ligands of different affinities, makes measurements of analyte concentrations possible, at levels of accuracy limited, in principle, only by the number of different ligand variants available, and by the affinity range of those variants.

The CD spectra of 1 μM solutions of KE2-P and KE3-P in 10 mM phosphate buffer at pH 7.5 revealed a higher degree of helical content than that of the template peptide LA-42b at comparable concentrations [Andersson, L. K.; Dolphin, G. T.; Kihlberg, J.; Baltzer, L. *J. Chem. Soc.-Perkin Trans. 2* **2000**, 459-464]. The mean residue ellipticity θ_{222} was -18 200 and -15 900 deg cm^2 $dmol^{-1}$ for 1 μM KE2-P and KE3-P, respectively, and that of 3 μM LA-42b was –7 500 deg cm^2 $dmol^{-1}$. The large difference between the KE-P peptides and LA-42b suggests that the probe has an effect on helix stability, possibly due to the removal of a positive charge upon probe attachment [Andersson, L. K.; Dolphin, G. T.; Kihlberg, J.; Baltzer, L. *J. Chem. Soc.-Perkin Trans. 2* **2000**, 459-464] or due to interactions between the probe and the hydrophobic

core. Difference CD spectra obtained by subtracting that of 2 μM HCAII from that of 1 μM KE2-PL and 2 μM HCAII showed that the helicity of KE2-PL was unchanged upon binding to HCAII, Figure 4. At these concentrations, and based on a dissociation constant of 0.02 μM, more than 90% of the peptide is bound to HCAII.

There was a significant difference between the fluorescence intensities of KE3-P and KE3-PL in the absence of HCAII. The attachment of a ligand in position 8 close to the probe in position 15 may change the peptide structure enough to decrease the quenching. This hypothesis is supported by the observed differences in helical contents between 1 μM KE3-P and 1 μM KE3-PL, θ_{222} = -15 900 and -21 300 deg cm^2 dmol^{-1}, respectively.

The affinity obtained from fluorescence spectroscopy was compared to that measured with a surface plasmon resonance-based method, where the interaction between immobilized HCAII and KE2-PL in solution was monitored for a series of peptide concentrations ranging from 40 nM to10 μM. The steady state affinity was estimated to be 0.08 μM from curve fitting to a plot of equilibrium responses as a function of peptide concentration using a 1:1 binding model. Slightly non-ideal behavior was observed, which might be explained by an influence on the affinity by concentration-dependent supersecondary structure formation of the peptide. The steady-state affinity of HCAII for **Ic** was also determined and K_d was found to be 0.044 μM. The results suggest that the affinity of HCAII for the ligand decreases due to steric effects when the ligand is attached to the peptide. An even longer spacer might be useful to increase the affinity of HCAII for the peptide even further.

It has been shown that components that have been designed to specifically recognize and bind an analyte and to signal this event via a reporter group can be introduced in a small synthetic molecule as part of a chemosensor. [Czarnik, A. W. *Chem. Biol.* **1995**, *2*, 423-428; Chen, C. T.; Wagner, H.; Still, W. C. *Science* **1998**, *279*, 851-853]. In a biosensor, the recognition event is based on a biochemical mechanism, involving e. g. antibodies, enzymes or whole cells [Thevenot, D. R.; Toth, K.; Durst, R. A.; Wilson, G. S. *Pure Appl. Chem.* **1999**, *71*, 2333-2348]. The polypeptide scaffolds according to the invention have been designed and synthesized and as they are further modified with ligands their functions resemble those of chemosensors. However, as the scaffolds themselves do not contribute to recognition, which is based on a biochemical mechanism, the inventors present the ligand-modified polypeptides,

with or without a reporter group, according to the inventions as functional units in biosensor systems.

These results demonstrate the first steps in the successful application of folded helix-loop-helix peptides to the area of biosensing. A peptide that binds to a receptor has been shown to be able to report on this event via a fluorescent probe. Furthermore, this concept has been applied to the determination of an affinity constant. The possibility of conveniently incorporating a wide range of probes and ligands at different relative positions provides an attractive way of optimizing the biosensing conditions, such as sensitivity and response, for any target biomacromolecule. As indicated by these results, the structure of the peptide scaffold also plays an important role in sensor performance. A determination of analyte concentration is possible using an array of peptides modified with ligands of different affinities.

CLAIMS

1. Polypeptide having a sequence according to SEQ. ID. No. 1, SEQ. ID. No. 2 and/or SEQ. ID. No. 3.

2. Polypeptide scaffold consisting of a four helix bundle formed of two dimerized helix-loop-helix motifs, said helix-loop-helix motifs having sequences independently selected from SEQ. ID. No. 1, SEQ. ID. No. 2 and SEQ. ID. No. 3.

3. Polypeptide scaffold according to claim 2 comprising an anchoring group for attachment of the polypeptide scaffold to a solid surface, wherein said anchoring group is either an available amino acid residue in the sequence of the polypeptide scaffold, or a group attached to at least one of the polypeptide chains forming the helix-loop-helix dimer.

4. Polypeptide scaffold according to claim 2 or 3 comprising in one or both of the polypeptide chains of the dimer a ligand with affinity for a target molecule or ion, and a reporter group which gives rise to a measurable signal upon binding of said ligand to said target molecule or ion.

5. Polypeptide scaffold according to claim 4 wherein said target molecule is a biomolecule.

6. Polypeptide scaffold according to claim 5 wherein said ligand is selected from the group consisting of peptides with affinity for a protein, proteins with affinity for a protein, inhibitors of an enzyme, agonists of a receptor protein, antagonists of a receptor protein, parts of DNA, parts of RNA, parts of PNA, carbohydrates, haptens, toxins, metabolites, transition state analogues, hormones, ion chelating agents, drugs, steroids, lipids and combinations of two or more of such ligands.

7. Polypeptide scaffold according to any one of the claims 4 - 6, wherein said reporter group is localized at the side chain of Lys15.

8. Polypeptide scaffold according to any one of the claims 4 - 7, wherein said reporter group is a fluorescent probe.

9. Polypeptide scaffold according to claim 8, wherein said fluorescent probe is selected from the group consisting of dansyl, fluorescein, rhodamin and nitrobensofurazan derivative.

10. Polypeptide scaffold according to any one of the claims 2 – 9 wherein both dimerized helix-loop-helix motifs have SEQ. ID. No. 2.

11. Polypeptide according to any one of the claims 2 – 9 wherein both dimerized helix-loop-helix motifs have SEQ. ID. No. 3.

12. Polypeptide scaffold according to any one of the claims 4 – 11 comprising at least one helix-loop-helix motif having SEQ. ID. No. 2, wherein said ligand with high affinity for a target molecule or ion is localized at the side chain of Lys34 in said at least one helix-loop-helix motif having SEQ. ID. No. 2.

13. Polypeptide scaffold according to any one of the claims 4 – 11, comprising at least one helix-loop-helix motif having SEQ. ID. No. 3, wherein said ligand with high affinity for a target molecule or ion is localized at the side chain of Lys8.

14. Polypeptide scaffold according to claim 12 or 13 wherein said ligand is benzenesulfonamide.

15. Polypeptide scaffold according to claim 3, wherein said anchoring group is introduced at position 22 by attachment to a Cys, Lys or Glu residue or a non-natural amino acid.

16. Polypeptide scaffold according to claim 15, wherein said non-natural amino acid has an aminooxy function.

17. Polypeptide scaffold according to claim 3, wherein said anchoring group has been introduced site-specifically by attachment of a bifunctional molecule to the accessible amino acid residues.

18. Polypeptide scaffold according to claim 17, wherein said bifunctional molecule has reacted with one of the amino acid residues in the polypeptide scaffold, and another group that forms a bond with a solid surface.

19. Polypeptide scaffold according to claim 17 or 18, wherein said bifunctional molecule has the general structure X-R_n-Y, wherein X is a functional group of the type COOH, NH_2, SH, SSAr, CHO, CH_2Br, CH_2Cl, or CH_2I, R_n is an alkyl or ethylene glycol chain comprising n carbons, and Y is a group of the type COOH, NH_2, SH, biotin, biotin analogue, His-tag, fatty acid or cholesterol.

20. Polypeptide scaffold according to claim 3, wherein said surface is the surface of a modified or unmodified metal, insulator, semiconductor or polymer.

21. Polypeptide scaffold according to claim 3 or 20, wherein said solid surface is modified with an organic coating that is a self-assembled monolayer, a protein layer, a polymer network, a hydrogel, or a his-tag.

22. Polypeptide scaffold according to claim 3, wherein said surface is the surface of a vesicle, a micelle or a membrane.

23. The use of at least two polypeptide scaffolds according to claims 3 and 4 or according to claims 22 and 4 in detection and/or analysis of a target molecule or ion, wherein all polypeptide scaffolds have the same affinity for the target molecule or ion.

24. The use of at least two polypeptide scaffolds according to claims 3 and 4 or according to claims 22 and 4 in detection and/or analysis of a target molecule or ion, wherein all polypeptide scaffolds have different affinities for the target molecule or ion.

25. The use according to claim 23 or 24, wherein said polypeptide scaffolds are present in a solution in the wells of a microtiter plate or in indentations on a solid surface.

26. The use according to claim 23 or 24, wherein said polypeptide scaffolds are present on a solid surface in the form of an array.

27. The use according to any one of the claims 23 – 26 in a fluorometric biosensor system for identification, characterization and/or quantification of peptides, proteins, DNA, RNA, PNA, metabolites, carbohydrates, drugs, steroids, and/or inorganic ions.

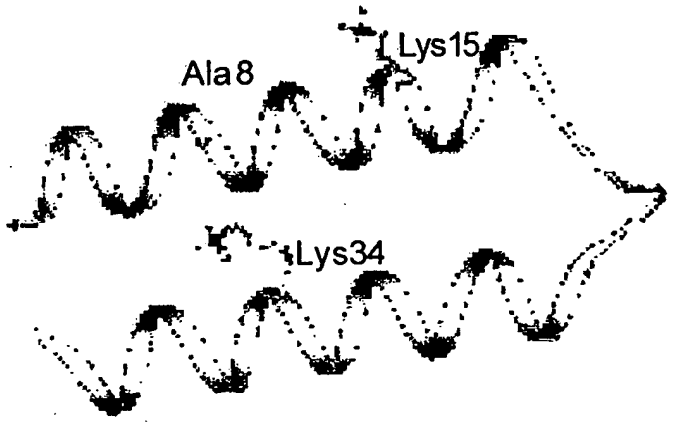
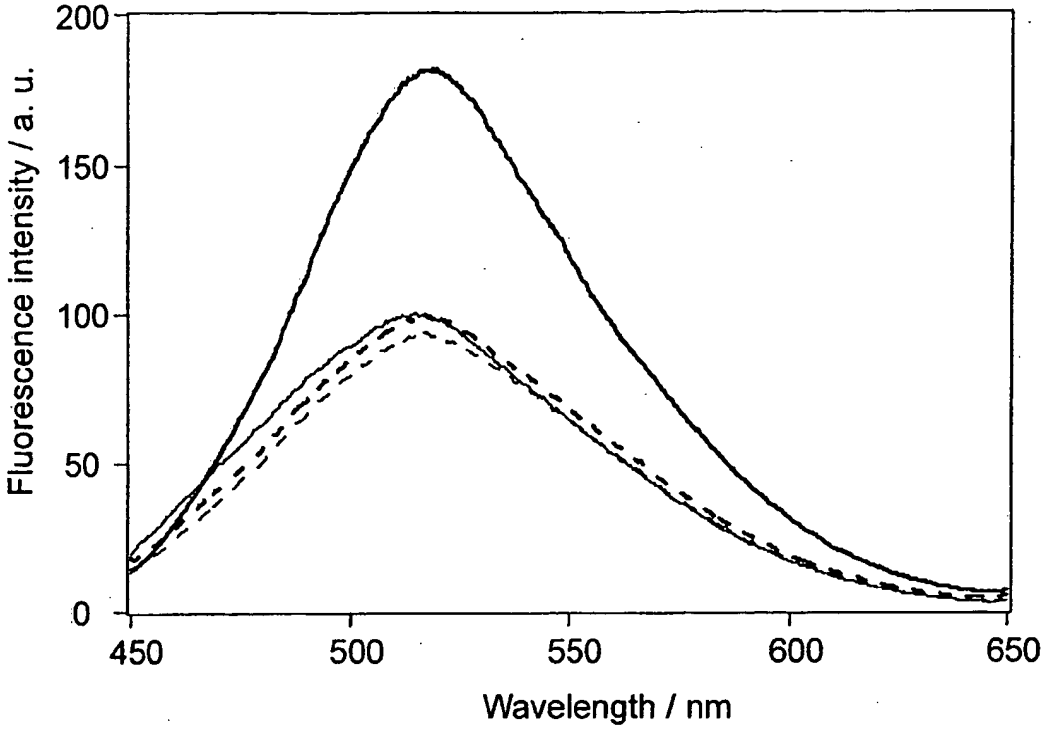


Fig. 1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Fluorescence intensity / a. u.
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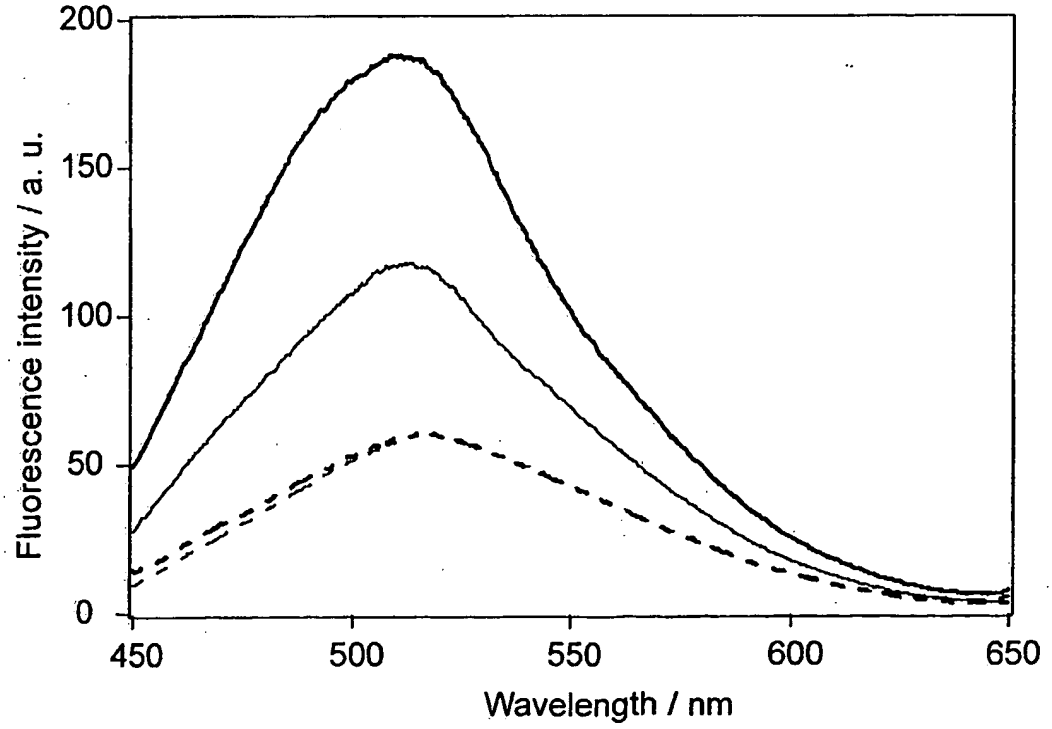
500
550
600
650
Wavelength / nm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Wavelength / nm

Fig. 2

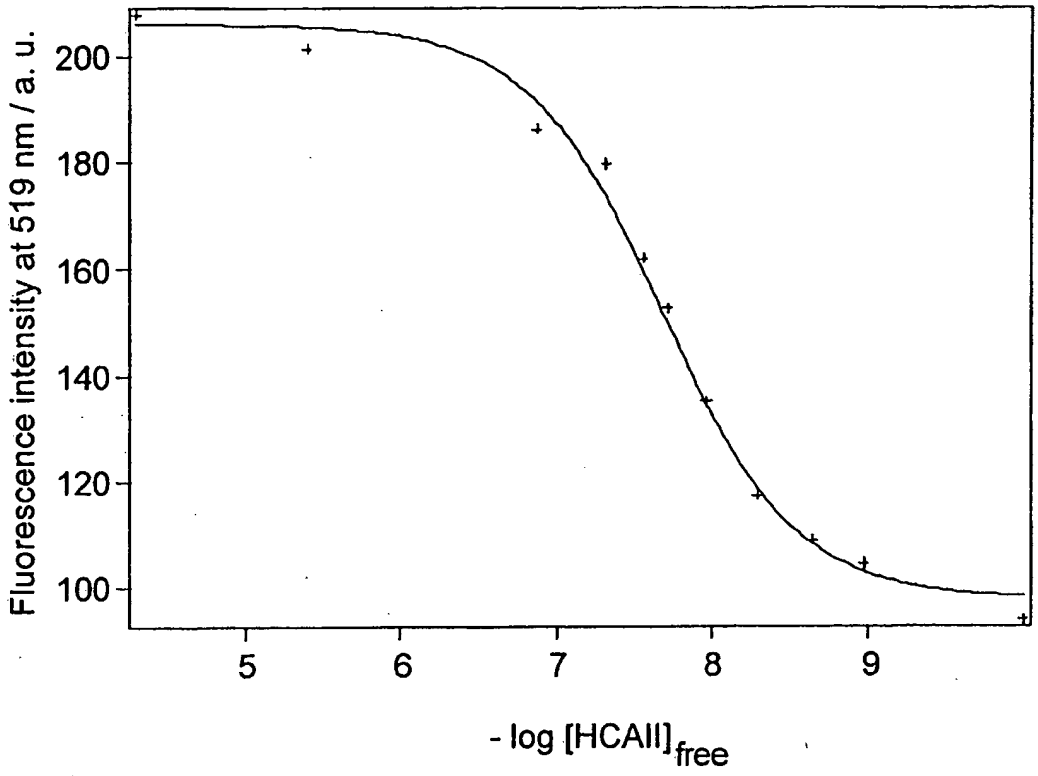
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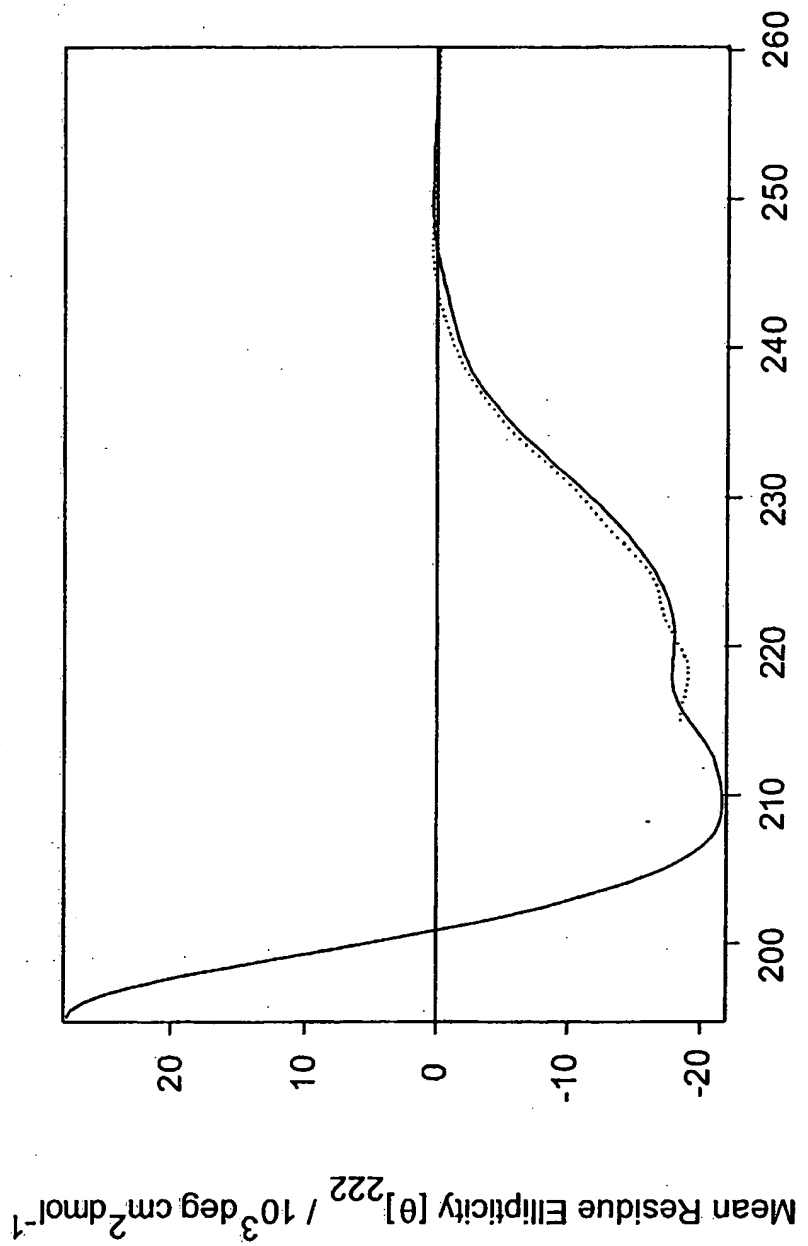
Fig. 3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Wavelength / nm

Fig. 4

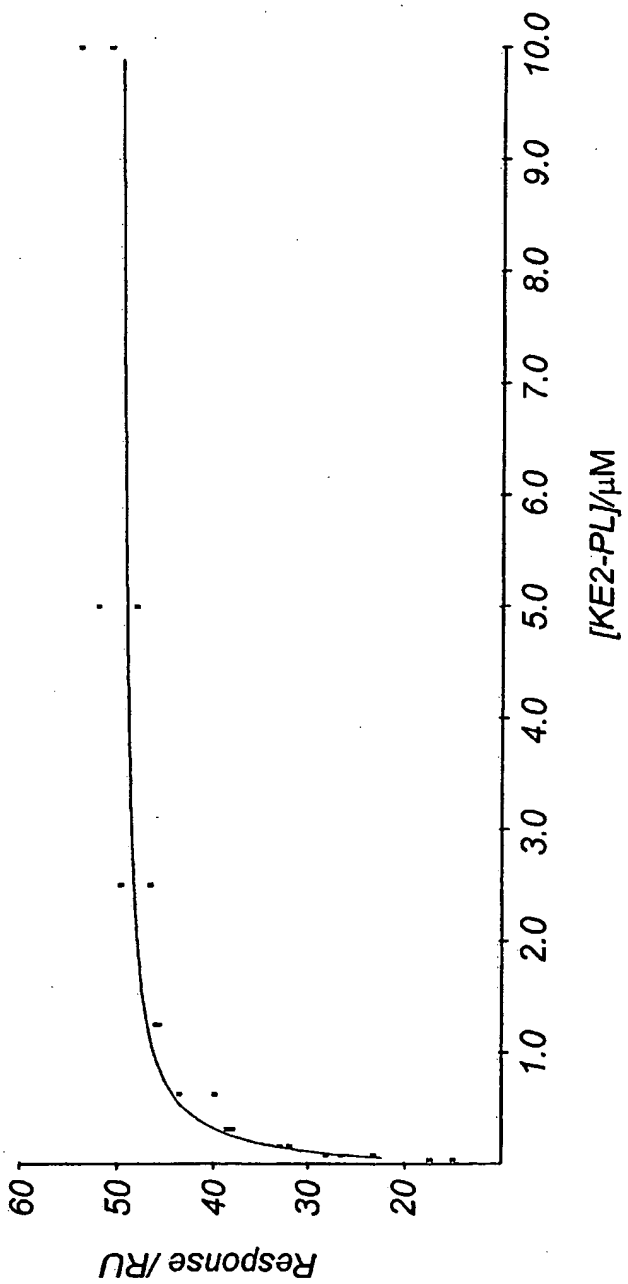


Fig. 5

KE2:

1 15 19
N-A-A-D-L-E-A-A-I-R-H-L-A-E-K-L-A-A-R
20
-G-P-V-D
42 34 24
G-A-R-A-F-A-E-F-K-K-A-L-Q-E-A-L-Q-A-A

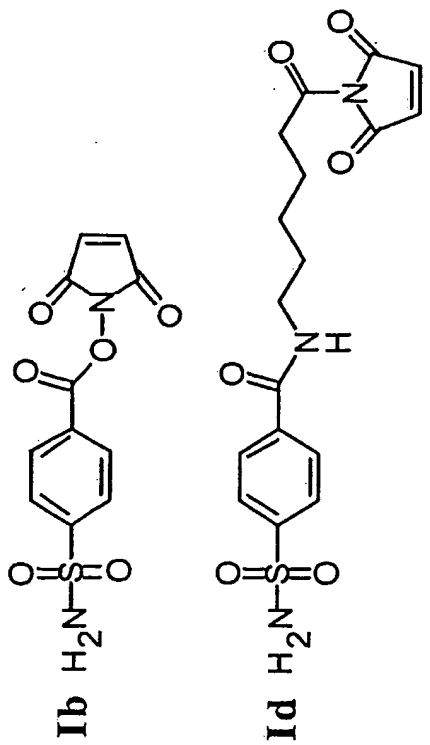
KE3:

1 8 15 19
N-A-A-D-L-E-A-K-I-R-H-L-A-E-K-L-A-A-R
20 23
-G-P-C-D
42 24
G-A-R-A-F-A-E-F-R-R-A-L-Q-E-A-L-Q-A-A

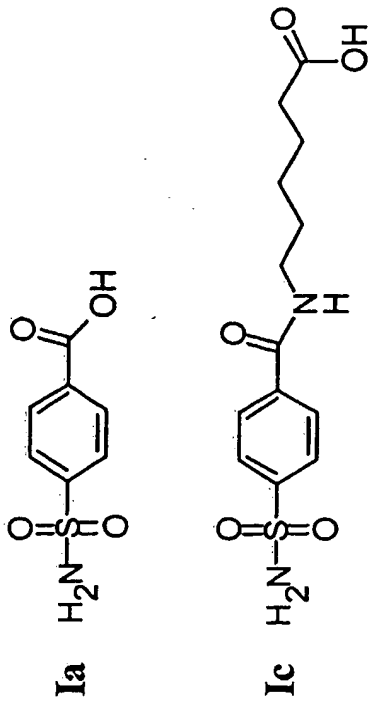
LA-42b:

1 19
N-A-A-D-Nle-E-A-A-I-K-H-L-A-E-K-Nle-A-A-K
20
-G-P-V-D
42 24
G-A-R-A-F-A-E-F-Orn-K-A-L-Q-E-A-Nle-Q-A-A

Fig. 6



Ib Id



Ia Ic

Fig. 7

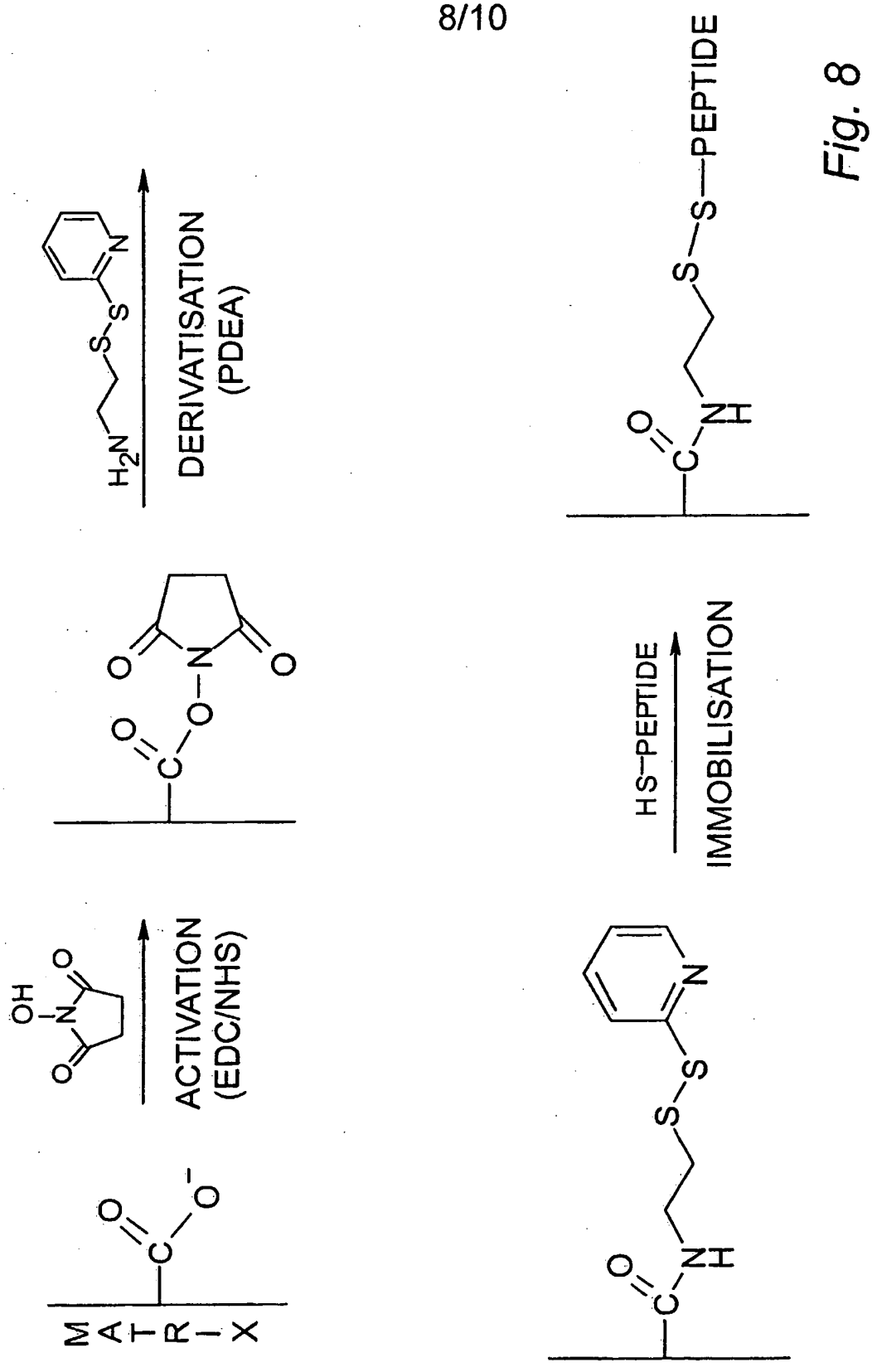
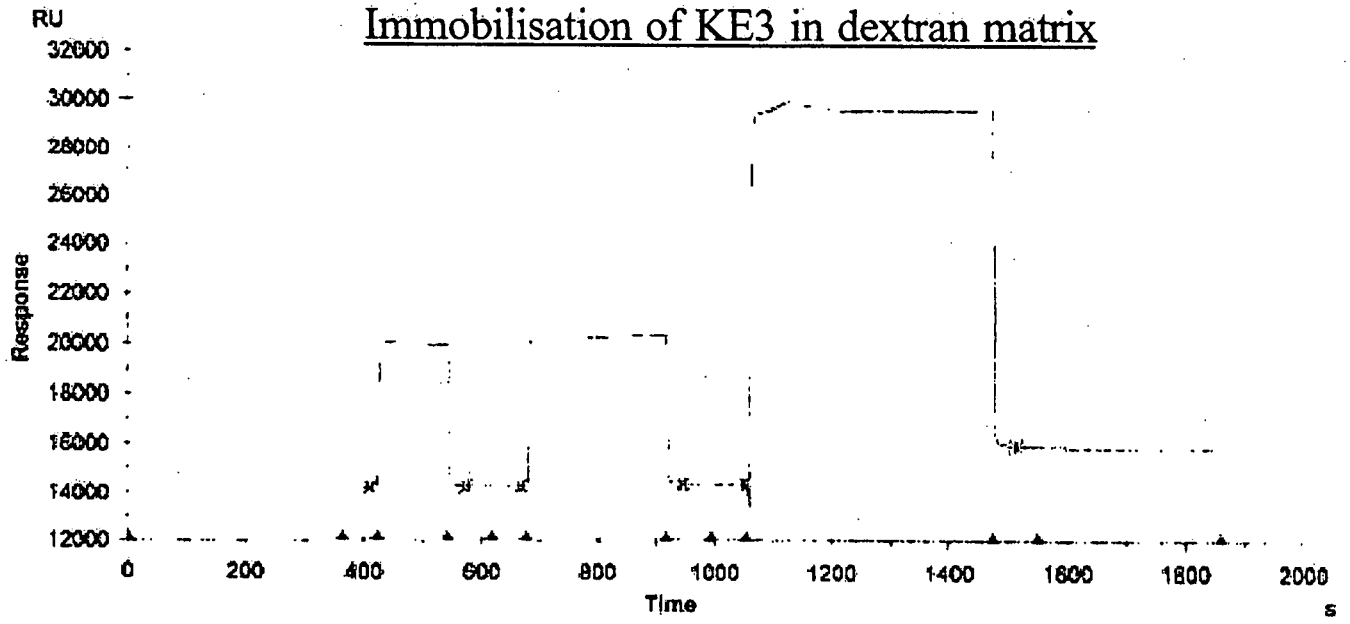

MATRIX
ACTIVATION (EDC/NHS)
DERIVATISATION (PDEA)
IMMOBILISATION
HS–PEPTIDE
S–S–PEPTIDE

Fig. 8

Immobilisation of KE3 in dextran matrix



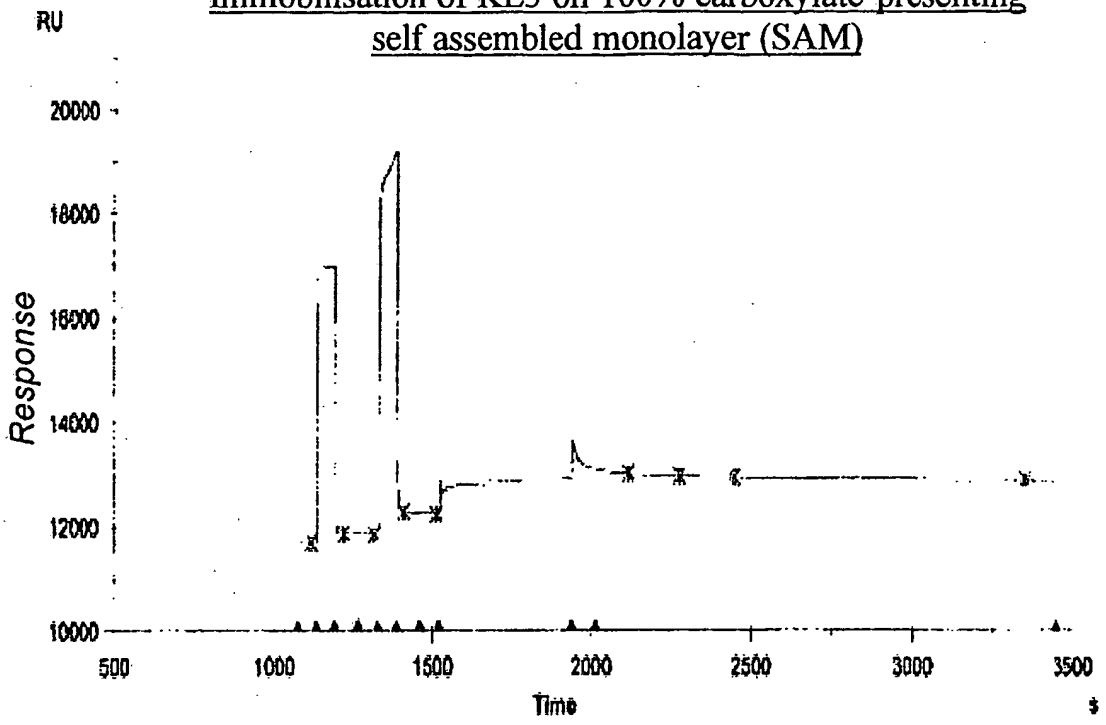
Fc	Time	AbsResp	Slope	Baseline	RelResp	Id
2	411.5	14215.1	-0.03	Yes	0	
2	576.5	14277.4	-0.34	No	62.3	aktivering EDC/NHS
2	668.5	14268.7	-0.03	No	53.6	
2	946.5	14419.9	-0.75	No	204.8	derivatisering PDEA
2	1049.5	14392.7	-0.05	Yes	177.5	
2	1513.5	15977.5	-1.17	No	1584.9	40 uM KE3 i acetat pH 5.0

activation 2 min
coupling of PDEA 4 min
coupling of KE3 7 min

[KE3] = 40µM in 10 mM Na-acetate buffer pH 5.0

Fig. 9

Immobilisation of KE3 on 100% carboxylate-presenting self assembled monolayer (SAM)



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1	1312.5	11858.9	-0.09	Yes	165.1	
1	1408.5	12287.4	-2.00	No	428.5	derivatisering PDEA
1	1512.5	12242.6	-0.06	Yes	383.7	
1	2117.5	13020.6	-0.45	No	778.0	40 uM KE3 i fosfat pH 6.0
1	2275.5	12975.6	-0.20	No	733.0	
1	2445.5	12951.3	-0.12	No	708.7	
1	3349.5	12901.2	-0.01	No	658.6	

activation 1 min
coupling of PDEA 1 min
coupling of KE3 7 min

[KE3] = 40μM in 10 mM phosphate buffer pH 6.0

Level of immobilisation: >600 RU
1 RU⇔1 pg/mm^2
If dimension of folded peptide is 20x20 Å^2, ~20-30 % of surface is covered by KE3

Fig. 9, cont.

SEQUENCE LISTING

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Ala Ala Lys Gly Pro Val Asp Ala Ala Gln Xaa Ala Glu Gln Leu Ala
20 25 30

Lys Xaa Phe Glu Ala Phe Ala Arg Ala Gly
35 40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 03/00507

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07K 14/00 // C07K 17/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI-DATA, EPO-INTERNAL, DIALOG, EBI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 0185906 A2 (A+ SCIENCE INVEST AB), 15 November 2001 (15.11.01), page 3, line 30 - page 4, line 13 --	1-27
A	WO 0185756 A2 (A+ SCIENCE INVEST AB), 15 November 2001 (15.11.01), page 3, line 34 - page 4, line 15, page 5, line 4, page 8, lines 6-37, page 9, lines 7-13, page 18, lines 3-9 --	1-27
A	Polymer Bulletin, Vol. 40, 1998, L. Scheibler et al: "Self-assembling functionalized templates in biosensor technology", page 151 - page 157, page 151, paragraph 1-3 av introduction --	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the interna date and not in conflict with the applicati the principle or theory underlying the inv
"X" document of particular relevance: the clai considered novel or cannot be considered step when the document is taken alone
"Y" document of particular relevance: the clai considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 July 2003	17 -07- 2003
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer patrick Andersson/BS Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 03/00507

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Journal of Molecular recognition, Vol. 13, 2000, Arne Skerra: "Engineered protein scaffolds for molecular recognition", page 167 - page 187 ---	1-27
P,X	J. Org. Chem., Volume 67, 2002, Karin Enander et al, "Designed, Folded Polypeptide Scaffolds That Combine Key Biosensing Events of Recognition and Reporting", pages 3120-3123 --- --------	1-27

INTERNATIONAL SEARCH REPORT

Information on patent family members

29/06/03

International application No.
PCT/SE 03/00507

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0185906 A2	15/11/01	AU 5691001 A	20/11/01
		AU 5896301 A	20/11/01
		AU 7464000 A	17/04/01
		EP 1272275 A	08/01/03
		EP 1283872 A	19/02/03
		EP 1283873 A	19/02/03
		JP 2003509664 T	11/03/03
		SE 0001698 D	00/00/00
		WO 0185756 A	15/11/01
WO 0185756 A2	15/11/01	AU 5691001 A	20/11/01
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